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**ORIGINAL RESEARCH PAPER**

# DEVELOPMENT OF AN EFFICIENT HPLC-DAD METHOD FOR DETERMINATION OF PRESERVATIVES IN DIFFERENT BEVERAGES IN ISFAHAN, IRAN

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**Abstract:** Preservatives are added to beverage formulations to prevent spoilage. In our study, an efficient method for the quantification of sodium benzoate (BEN) and potassium sorbate (SOR) in beverages was applied using reversed-phase high-performance liquid chromatography (RP-HPLC) with diode array detection (DAD). Ninety-seven samples were collected in two categories (carbonated and non-carbonated drinks) from the Isfahan market, Iran. Under optimal conditions, the retention times for determining the preservatives were less than 5 minutes. Among all samples, 6.1 % and 5.1 % of non-carbonated drinks contained BEN and SOR, respectively, which are not allowed according to Iranian standards. Additionally, all concentrations of BEN and SOR were lower than the general standards of the Iran National Standards Organization (600 $mg \cdot L^{-1}$).

**Keywords:** *beverages, HPLC, potassium sorbate, preservatives, sodium benzoate*

## INTRODUCTION

With the increasing world population, the demand for food products is growing, making the study of nature and quantity of additives in beverages a global health issue. The food industry applies various additives such as sweeteners, colors, and preservatives to carbonated and non-carbonated drinks to enhance their flavor, shelf-life stability, quality, appearance, and consistency, thereby making the product economical [1].
Preservatives are most used in beverages to protect them against spoilage from organisms and maintain the odor, taste, and appearance [2]. Some widely used food preservatives are benzoic acid, sorbic acid, their salts, sodium chloride, sulfites, and acetic acid [3]. In most countries, preservatives are allowed to be used based on the law, with some rules regulating the highest acceptable ranges of preservatives that may cause damage to different parts of the human body such as the liver, kidney, skin, pancreas, and heart [4]. Among preservatives, sodium benzoate (BEN) and potassium sorbate (SOR) are commonly used in beverages, which are effective in acidic media and kill different microorganisms [5]. Despite the different benefits of BEN and SOR, these preservatives have some problems such as hepatic failure [6, 7], kidney damage [7, 8], cancer risk [9 – 11] and Parkinson's disease [12]. It is essential for consumers to understand the potential risks associated with these preservatives, enabling them to make informed decisions about their beverage choices. In different countries, quantifying the amount of preservatives has become a focus due to their overuse and harmful potential [13]. Thus, based on the Iranian regulations (INSO 1250), the maximum permitted levels for SOR and BEN in carbonated drinks are 500 and 150 $mg \cdot kg^{-1}$, respectively, and they are banned in other beverages and foodstuffs [14]. Also, the maximum acceptable ranges in food products according to European regulation and Codex standards are 150 $mg \cdot kg^{-1}$ for BEN and 300 $mg \cdot kg^{-1}$ for SOR [2]. Up to now, different analytical methods have been used to determine preservatives and other additives in food products. Chromatographic methods such as high-performance liquid chromatography [15, 16], gas chromatography (GC) [17, 18], ion chromatography [19, 20], and spectroscopic techniques such as Infrared Spectroscopy (FTIR) [21], and UV/Vis Spectroscopy are primarily used in the determination of preservatives [22 – 24]. Recently Szekelyhidi *et al*. optimized a HPLC-DAD method for the determination of preservatives (SOR, BEN) in 69 beverages in the Hungarian market [25]. In another work, Yazdanfar *et al*. tested BEN and SOR in different sauce samples using the HPLC-DAD method [13].
In the present work, RPHPLC-DAD method was applied to quantify of BEN and SOR in different beverages (97 samples) available on the Isfahan markets in Iran. All the results were compared to the maximum permitted level in Regulation No. 1250 of the Iranian National Standardization Organization. These preservatives were selected because they are commonly used in beverages, and HPLC-DAD was applied as a fast, simple, and available method for many QC departments and laboratories.

## MATERIALS AND METHODS

### Chemicals

All chemicals were purchased from Merck Chemical, Germany, in HPLC grade. Ammonium acetate (99 %), acetonitrile (ACN) (≥ 99.9 %), acetic acid (100 %), methanol

(MeOH) (> 99.8 %), and ultra-pure water were used without any purification. Analytical standards of sodium benzoate (99.6 %) and potassium sorbate (99.4 %) were purchased from Sigma-Aldrich Chemical Company. HPLC analysis was performed with an Agilent 1260 Infinity II series composed of a diode array detector (DAD) (Agilent Technologies, Germany), a tertiary pump, an autosampler with degasser. Analytes were separated using Adamas C18-Extreme (250 x 4.6 mm, 5 µm) column (SepaChrom, Italy). The Open Lab 3.5 software (Agilent Technologies, Germany) was used to analyze the results. Also, a *p*H meter Metrohm (827, Switzerland), an ultrasonic bath Elma (Iran), a vacuum filtration system (JUN-AIR, Denmark) and Whatman membrane filters (0.45 µm pore size) were used.

**Sample collection preparations**

All samples from different brands (n = 97) were collected from the Isfahan market in Iran. They were categorized into two groups: carbonated and non-carbonated. Samples were stored in a refrigerator until analysis. The preparation of samples in this study was straightforward. Carbonated samples were degassed using an ultrasound bath for 15 minutes. Subsequently, 5 mL of each sample was transferred into a 50 mL volumetric flask and diluted to the mark with a buffer solution. Non-carbonated semi-solid samples were homogenized in a blender for 5 minutes. Then, 5 g of each sample was transferred into a 50 mL volumetric flask, and acetate buffer was added to reach a total volume of 50 mL. All samples were filtered with a 0.45 µm syringe filter directly into the sample vial.

**Apparatus and chromatographic conditions**

HPLC analysis was performed using an Agilent 1260 Infinity II series that included a diode array detector (DAD), a tertiary pump, and an autosampler with a degasser. Analytes were separated using an Adamas C18-Extreme (250 × 4.6 mm, 5 µm) column at a constant column temperature of 25 °C. Acetonitrile (30 mL) combined with acetate buffer (70 mL) under isocratic conditions was selected as the mobile phase at a flow rate of 1 $mL \cdot min^{-1}$. The wavelength of the detector was set at 225 nm for sodium benzoate and 250 nm for potassium sorbate. Using the autosampler, 20 µL of each sample was injected into the HPLC operating system. Each examination was obtained in triplicate, and the average values of peak areas were taken.

**Preparation of standard and buffer solutions**

Stock solution was prepared by dissolving 100 mg of each preservative in a 100 mL volumetric flask and adding water to bring the total volume up to 100 mL. This solution contains 1000 $mg \cdot L^{-1}$ of each preservative and was stored at 4 °C. Calibration standard solutions (1.56, 3.12, 6.25, 12.5, 25, and 50 ppm) were prepared by diluting the stock solution in acetate buffer. Working standard solutions were prepared daily. The buffer solution was prepared by dissolving 0.3 g of ammonium acetate in 1000 mL of high-purity HPLC grade water. Then, the *p*H was adjusted to 4.2 with acetic acid. The buffer solution was filtered using a 0.45 µm membrane filter under reduced pressure.

## RESULTS AND DISCUSSION

A chromatographic method was developed and optimized for the separation of BEN and SOR in beverage matrices. For this purpose, a reverse phase C18 column was chosen to provide satisfactory separation, and it can be used with samples at different *p*H values (2 - 9) [25]. A UV-DAD detector was used because it has good sensitivity at mg·L$^{-1}$ levels for the determination of preservatives [25]. To find the maximum sensitivity for the quantification of the preservatives, the determination was examined at wavelengths of 220, 225, 230, 235, and 250 nm. The wavelengths were adjusted to around 225 nm for BEN and 250 nm for SOR. According to the literature, the best separation for BEN and SOR occurs in organic solvents with a mobile phase consisting of an acetate buffer (*p*H 2.4 - 4.2) [13, 15, 16]. In this study, acetonitrile was investigated as an organic solvent with a low back pressure and a lower UV cutoff wavelength (190 nm), which is most used for quantifying preservatives [26]. Different mobile phases with various *p*H levels were examined for the best optimized elution program. Initially, the *p*H values (2, 4, 5, 6, and 7) were tested, and the best resolution was obtained in acetate buffer with *p*H 4. Secondly, the nature of the mobile phase was tested. Several mobile phases consisting of methanol, acetonitrile, and a mixture of acetate buffer were tested. Based on optimal separation conditions, the mobile phase consisted of acetonitrile and acetate buffer (*p*H 4.2) in a 30:70 volume ratio (V/V) under isocratic conditions. To decrease the HPLC run time, the flow rate was evaluated between 0.5 - 2.5 mL·min$^{-1}$, and the best resolution was observed at a flow rate of 1 mL·min$^{-1}$. Under the optimized flow rate, retention times of 5 and 6.2 minutes were obtained for sodium benzoate and potassium sorbate, respectively. Under the conditions found above, chromatograms (Figure 1) of BEN and SOR in a blank sample with two spiked concentrations of 100 and 300 mg·L$^{-1}$ at 225 and 250 nm were obtained.

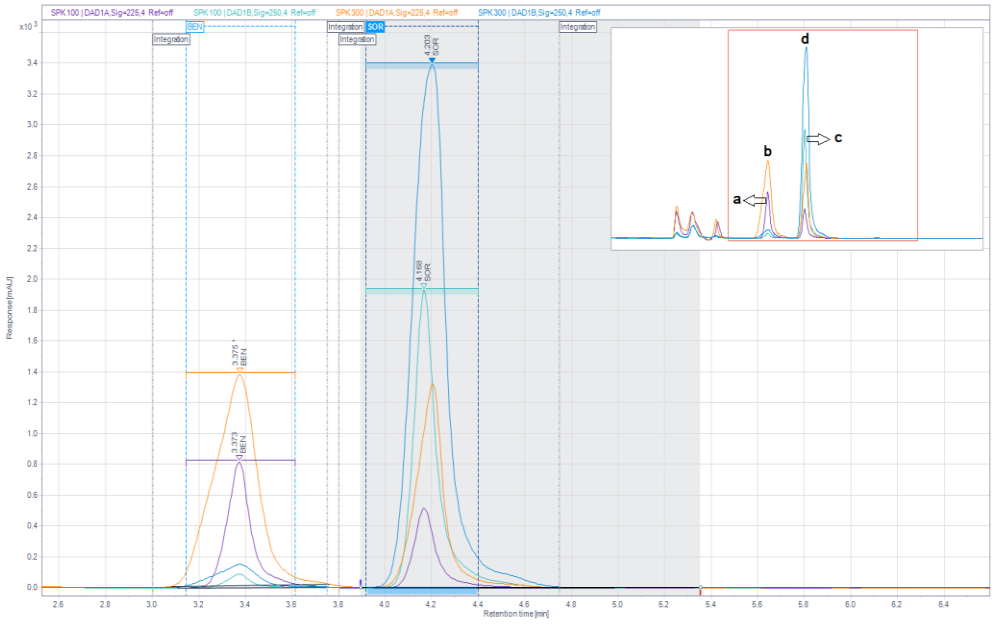


***Figure 1.*** *The HPLC-DAD chromatograms of sodium benzoate (a and b) and potassium sorbate (c and d) in a blank sample with two spiked concentrations of 100 and 300 mg·L$^{-1}$*

As illustrated in Figure 1, the preservatives were detected at the following retention times: 3.3 minutes for BEN and 4.2 minutes for SOR under optimized conditions.
Additionally, the spectrometric data for BEN and SOR were illustrated using isoabsorbance plots (Figure 2).

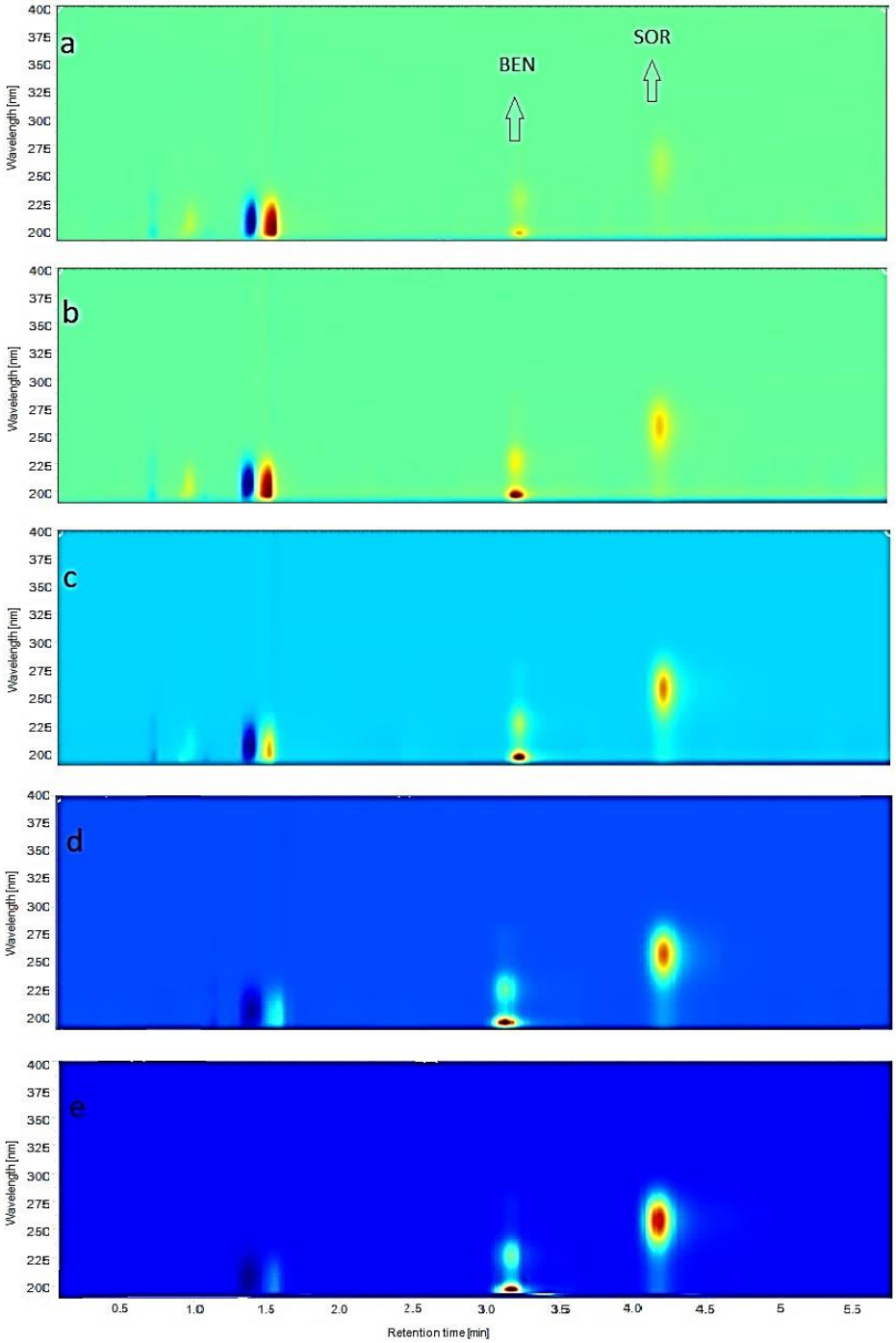


***Figure 2.*** *Isoabsorbance plots of BEN and SOR at different concentrations (3.12, 6.25, 12.5, 25 and 50 mg·L$^{-1}$)*

These plots were compiled with retention time on the x-axis and wavelength on the y-axis. As shown in Figure 2, with increasing concentration and absorbance intensity, the color of the plots transitions from light green (plot a) to dark blue (plot e). According to Figure 2, the spots for BEN and SOR were detected at approximately 3.3 minutes and 4.2 minutes, respectively.

## Linearity

For the linearity study, six concentration levels of BEN and SOR (1.56, 3.12, 6.25, 12.5, 25, and 50 mg·L$^{-1}$) were used, and calibration curves were created (Figures 3 and 4). The calibration curves were plotted based on peak areas and different concentrations of BEN and SOR. Using the least squares regression method, calibration equations and correlation coefficients ($R^2$) of the preservatives were determined. All the data obtained are summarized in Table 1. According to the coefficient values of the standard curves (greater than 0.9999), the developed process demonstrated appropriate linearity of the analytical response at the examined concentration levels.

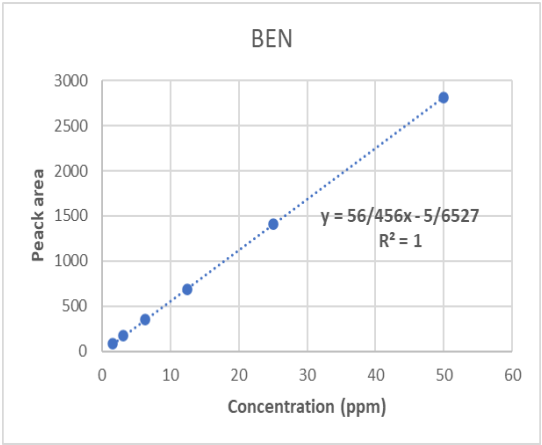


***Figure 3.*** *Calibration curve of sodium benzoate of DAD-HPLC*

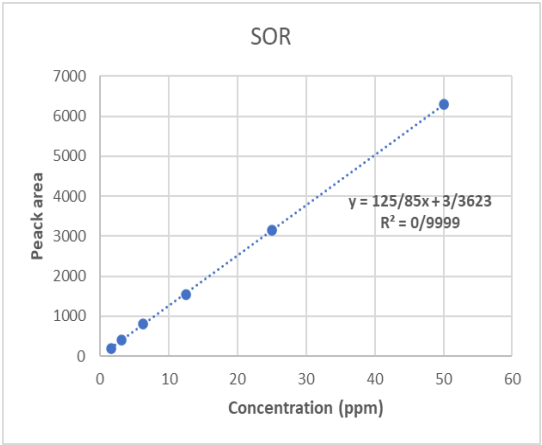


***Figure 4.*** *Calibration curve of potassium sorbate of DAD-HPLC*

## Sensitivity

The lowest concentration of the analyte that is measurable by instrument is defined as the limit of detection (LOD), and the lowest concentration of the analyte that can be quantified with appropriate precision and accuracy is defined as the limit of quantification (LOQ). The outcomes are listed in Table 1.

***Table 1.*** *Linearity and sensitivity obtained from the standard solutions of preservatives (n = 3)*

| Analyst | Calibration equations | Linear range [mg·L$^{-1}$] | $R^2$ | LOD [mg·L$^{-1}$] | LOQ [mg·L$^{-1}$] |
|---|---|---|---|---|---|
| **BEN** | Y=56.456x-5.6527 | 1.56-50 | 1 | 0.4 | 1.4 |
| **SOR** | Y=125.85x-3.3623 | 1.56-50 | 0.9999 | 0.4 | 1.5 |

Based on the data in Table 1, the LOD values of preservatives were 0.4 ppm for both SOR and BEN, and the LOQ values were found to be 1.4 and 1.5 ppm for BEN and SOR, respectively. In this case, the method was sufficiently sensitive to quantify each analyte.

**Precision**

Intra-day precision was investigated to determine the method's repeatability (Table 2). For this purpose, BEN and SOR were spiked into a fresh sample at a concentration of 20 $mg \cdot L^{-1}$ and injected five times each. Peak areas and concentrations were recorded for all the peaks. The relative standard deviations (RSD) of the concentrations were calculated to express the intra-day precision. The calculated RSDs % with the developed method were 1.49 % and 1.84 % for BEN and SOR, respectively. The RSD % of intra-day was less than 2 percent, indicating that the intra-day precision of the developed method was sufficient to quantify the analytes.

***Table 2.*** *Intra-day precision of tested preservatives*

| | **BEN** | | **SOR** | |
|---|---|---|---|---|
| **Concentration [$mg \cdot L^{-1}$]** | **Found Mean ± SD** | **RSD [%] Intraday (n=5)** | **Found Mean ± SD** | **RSD [%] Intraday (n=5)** |
| 20 | 20/44 ± 0.3 | 1.49 | 20.99 ±0.38 | 1.84 |

**Accuracy**

The accuracy of the developed method was studied by determining the recovery percentage. For this purpose, sodium benzoate and potassium sorbate were spiked into a preservative-free sample at concentrations of 100 and 300 $mg \cdot L^{-1}$. Analyte addition was repeated three times and data were collected in Table 3.

***Table 3.*** *The results of the accuracy test*

| **Preservatives** | **Spiked Conc. [$mg \cdot L^{-1}$]** | **Recoveries [%]** |
|---|---|---|
| **BEN** | 100 | 106.8 |
| | 300 | 104.3 |
| **SOR** | 100 | 104.07 |
| | 300 | 86.4 |

According to Table 3, recovery percentages were 104.3 % - 106.8 % in apple for BEN and 86.4 % - 104.07 % for SOR. The recoveries obtained demonstrated that the method used showed good recovery, and in most cases, were near 100 %, indicating that the developed method was suitably accurate.

**Sample analysis**

In continuation of our previous works about the analysis of foodstuffs using chromatographic methods [27 – 30], we developed a DAD-HPLC method to determine preservatives (BEN and SOR) in different beverages (97 samples) in the Isfahan market. There have been some published works to determine sodium benzoate and potassium sorbate in beverages in Iranian markets. In a comprehensive study, Amirpour and co-

workers [31] investigated the preservatives in foodstuffs in Sari, Iran. According to their findings, the maximum levels of BEN and SOR were 114 and 68 mg·kg$^{-1}$ in soft carbonated drinks, which were within the acceptable range according to IRIRI. In another work, Khosrokhavar *et al*. [15] collected 50 samples of soft drinks from the Tehran market, and their preservatives were quantified. The mean concentrations of BEN in the carbonated apple beverages were found to be 163 mg·L$^{-1}$, which were higher than the general standard of the ISIRI, and for SOR were 316 mg·L$^{-1}$, which were within the acceptable range based on ISIRI legislation. The other detected beverages are allowed according to the ISIRI. Our findings were collected in Table 4.

***Table 4.*** *Concentration (mg·L$^{-1}$) of preservatives (BEN and SOR) in the pooled beverages*

| Samples | Sample details | BEN [mg·L$^{-1}$] [1] | SOR [mg·L$^{-1}$] |
|---|---|---|---|
| S1 | Black carbonated drink | 119.8 | 167.3 |
| S2 | Carbonated drink with orange flavour | ND | 160.9 |
| S3 | Carbonated drink with lemon flavour | 88.0 | ND |
| S4 | Carbonated with orange flavour | 63 | ND |
| S5 | Black carbonated drink | 75.7 | ND |
| S6 | Carbonated drink with mango flavour | 43 | ND |
| S7 | Carbonated drink with mojito flavour | 71.1 | ND |
| S8 | Carbonated drink with stevia flavour | 117.7 | ND |
| S9 | Carbonated drink with grape flavour | 98.0 | ND |
| S10 | Carbonated drink with lemon flavour | 93.0 | 4.8 |
| S11 | Non-carbonated with Pineapple flavour | ND | 5.7 |
| S12 | Non-carbonated drink with coconut flavour | ND | 38.2 |
| S13 | Carbonated drink with watermelon flavour | 14.6 | ND |
| S14 | Non-carbonated drink with cherry flavour | 4.7 | ND |
| S15 | Non-carbonated drink with mango flavour | 103.7 | ND |
| S16 | Non-carbonated drink with mango flavour | 3.9 | ND |
| S17 | Non-carbonated drink with orange flavour | 88.4 | ND |
| S18 | Carbonated drink with mulberry flavour | 149.6 | ND |
| S19 | Non-carbonated drink with peach flavour | 24.5 | 79.5 |
| S20 | Non-carbonated drink with aloevera flavour | 87.4 | 94.3 |
| S21 | Non-carbonated drink with aloevera flavour | ND | 151.1 |
| S22 | Carbonated drink with watermelon flavour | ND | 6 |
| S23 | Carbonated drink with grape flavour | 140.8 | ND |
| S24 | Carbonated drink with lemon flavour | 56.7 | ND |

[1] Concentrations were reported by applying the dilution factor
ND = Not detected

According to Regulation No 1250 of the Iranian National Standardization Organization [14], the use of SOR and BEN in carbonated beverages is allowed, while other non-carbonated drinks are forbidden. According to this regulation, the maximum allowable levels for SOR and BEN are 500 and 150 mg·L$^{-1}$, respectively. Among all the samples, 24 contained sorbate or benzoate, and all tested samples were within the allowable range. Also, 19.5 % (19 out of 97 samples) of tested drinks contained sodium benzoate at concentrations ranging from 3.9 to 149.6 mg·L$^{-1}$ and were within the allowable range (< 150 mg·L$^{-1}$). Additionally, 9.2 % (9 out of 97 samples) of beverages contained

potassium sorbate and were observed at ranges from 4.8 to 167.3 mg·L$^{-1}$, which were within the permitted level (< 500 mg·L$^{-1}$). Among all samples, 6.1 % and 5.1 % of non-carbonated drinks contained BEN and SOR, respectively, which are not allowed based on the Iranian standard.

Our developed method was compared with earlier published HPLC methods in terms of retention time, RSD, and LOQ for quantification of sodium benzoate and was summarized in Table 5. In most cases, the LOQ parameter of our study (1.49 mg·L$^{-1}$) was better compared to the other published works (LOQs were between 1.39-13.7 mg·L$^{-1}$) [25, 32 – 36]. Furthermore, our developed method was shorter in most cases when compared to some other methods (retention time: 5 min). Finally, the developed approach was suitable for the separation and quantification of preservatives.

***Table 5.*** *A comparison study of our developed DAD-HPLC method with some previously published studies*

| Entry | Determination / separation technique | Mobile phase | Run time [min] | RSD [%] | LOQ [mg·L$^{-1}$] | References |
|---|---|---|---|---|---|---|
| 1 | UV / RP-HPLC | Acetonitrile-ammonium acetate buffer (*p*H 3.6) | - | 4.08 | 6.61 | [35] |
| 2 | DAD / RP-HPLC | Acetonitrile- phosphate buffer (*p*H 3.3) | 7.34 | 0.34 | 1.39 | [25] |
| 3 | UV / RP-HPLC | Acetonitrile- phosphate buffer (*p*H 3) | 2.29 | 0.02 | 13.7 | [33] |
| 4 | UV / RP-HPLC | Methanol–acetate buffer (*p*H 4.2) | 7.67 | 3.81 | 2.65 | [36] |
| 5 | UV / RP-HPLC | Acetonitrile-sodium ammonium acetate buffer (*p*H 4.3) | 4.9 | 1.83 | 1.8 | [34] |
| 6 | UV / RP-HPLC | Methanol-ammonium acetate buffer (*p*H 4.74) | 10.2 | - | 5.6 | [32] |
| 7 | UV / RP-HPLC | Acetonitrile-sodium ammonium acetate buffer (*p*H 4.2) | 7.4 | 0.9 | 1.9 | [31] |
| 8 | DAD / RP-HPLC | Acetonitrile-ammonium acetate buffer | 3.3 | 2.1 | 1.49 | Current work |

## CONCLUSION

Regardless of the extreme use of preservatives in the food industry and their associated hazard problems, the permitted level of the preservatives could protect foodstuff from decomposition. Thus, monitoring of the preservatives in foodstuff is essential for user health and financial aspects. In the present study, the quantity of the preservatives, namely sodium benzoate and potassium sorbate, was determined in beverages in the Isfahan market. Among all the samples, 24 out of the samples contained BEN or SOR, and all tested samples were in permissible level. Also, 19.5 % (19 out of 97 samples) of tested samples contained BEN at concentrations ranging from 3.9 to 149.6 mg·L$^{-1}$ and were in allowable range (< 150 mg·L$^{-1}$). Furthermore, in 9.2 % (9 out of 97 samples) of beverages, SOR was observed ranging from 4.8 to 167.3 mg·L$^{-1}$, which were in allowed range (< 500 mg·L$^{-1}$). Most of the samples showed acceptable levels of preservatives except

about 6.1 % and 5.1 % of non-carbonated drinks contained BEN and SOR respectively, which are not permitted based on Iranian standards. Consequently, periodical and careful supervision of these preservatives in the beverages is proposed to provide user health.

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